Video Article

A Simple Chelex Protocol for DNA Extraction from *Anopheles* spp.

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Abstract

Endemic countries are increasingly adopting molecular tools for efficient typing, identification and surveillance against malaria parasites and vector mosquitoes, as an integral part of their control programs[1,2,3,4,5]. For sustainable establishment of these accurate approaches in operations research to strengthen malaria control and elimination efforts, simple and affordable methods, with parsimonious reagent and equipment requirements are essential[6,7,8]. Here we present a simple Chelex-based technique for extracting malaria parasite and vector DNA from field collected mosquito specimens.

We morphologically identified 72 *Anopheles gambiae sl.* from 156 mosquitoes captured by pyrethrum spray catches in sleeping rooms of households within a 2,000 km^2 vicinity of the Malaria Institute at Macha. After dissection to separate the head and thorax from the abdomen for all 72 *Anopheles gambiae sl.* mosquitoes, the two sections were individually placed in 1.5 ml microcentrifuge tubes and submerged in 20 µl of deionized water. Using a sterile pipette tip, each mosquito section was separately homogenized to a uniform suspension in the deionized water. Of the ensuing homogenate from each mosquito section, 10 µl was retained while the other 10 µl was transferred to a separate autoclaved 1.5 ml tube. The separate aliquots were subjected to DNA extraction by either the simplified Chelex or the standard salting out extraction protocol[9,10]. The salting out protocol is so-called and widely used because it employs high salt concentrations in lieu of hazardous organic solvents (such as phenol and chloroform) for the protein precipitation step during DNA extraction[9].

Extracts were used as templates for PCR amplification using primers targeting arthropod mitochondrial nicotinamide adenine dinucleotide dehydrogenase (NADH) subunit 4 gene (ND4) to check DNA quality[11], a PCR for identification of *Anopheles gambiae* sibling species[10] and a nested PCR for typing of *Plasmodium falciparum* infection[12]. Comparison using DNA quality (ND4) PCR showed 93% sensitivity and 82% specificity for the Chelex approach relative to the established salting out protocol. Corresponding values of sensitivity and specificity were 100% and 78%, respectively, using sibling species identification PCR and 92% and 80%, respectively for *P. falciparum* detection PCR. There were no significant differences in proportion of samples giving amplicon signal with the Chelex or the regular salting out protocol across all three PCR applications. The Chelex approach required three simple reagents and 37 min to complete, while the salting out protocol entailed 10 different reagents and 2 hr and 47 min' processing time, including an overnight step. Our results show that the Chelex method is comparable to the existing salting out extraction and can be substituted as a simple and sustainable approach in resource-limited settings where a constant reagent supply chain is often difficult to maintain.

Video Link

The video component of this article can be found at http://www.jove.com/video/3281/

Protocol

1. Preparatory Dissection of Mosquito Specimens

1. Place mosquito on a small cutting of parafilm and mount on dissecting microscope.
2. Cover in drop of deionized water to soften tissue.
3. Incise the mosquito carcass precisely at the joint between the thorax and abdomen, thus nicking off the head and thorax from the abdominal section.
4. Transfer each dissected mosquito section into a clean autoclaved 1.5 ml microcentrifuge tube prelabeled with mosquito ID number details and appropriately marked to denote body section (*e.g.* "m" for mid-gut section; "ht" for head and thorax section).
5. Discard parafilm and carefully wipe microscope stage with 70% alcohol-moistened Kimwipe before processing the next mosquito.

2. DNA Extraction from Mosquito Specimens

1. Pipette 20 µl of deionized water into sample tube and use pipette tip to grind the submerged mosquito section into a uniform suspension.
2. Add 100 µl of autoclaved 1X PBS/1% saponin solution to sample homogenate and mix by gentle momentary vortexing.
3. Incubate at room temperature for 20 min.
4. Centrifuge at 20,000 x g for 2 min and discard supernatant.
5. Resuspend pellet in 100 µl 1X PBS.
6. Centrifuge again at 20,000 x g for 2 min and discard supernatant.
7. By gentle vortexing (5 sec), resuspend pellet in 75 µl sterile deionized water and 25 µl of 20% w/v Chelex-100 resin suspension in deionized water.
8. Pierce hole in lid of sample tube using sterile 23G hypodermic needle flamed in Bunsen burner.
9. Boil sample suspension in water bath on floating rack (or in heating block) for 10 min.
10. Centrifuge at 20,000 x g, for 1 min and transfer ensuing DNA solution into prelabeled storage vial for use as template in PCR applications.

3. Plasmodium falciparum Genotyping and *Anopheles spp.* Molecular Identification

1. Add 2.5 µl of the Chelex DNA extract in 25 µl PCR reactions to check DNA quality[11], identify *Anopheles* species[10] and to genotype *P. falciparum*[12]mid-gut and salivary gland infections.
2. To maximize amplicon yield, especially for *P. falciparum* detection, include 1.5X bovine serum albumin (BSA) in the PCR assay. The following reaction composition is recommended: (2.5 µl template, 0.25 µM primers, 1.5 µM magnesium chloride, 200 µM dNTPs, 1X PCR Buffer, 1.5X BSA and 1.0U Taq DNA polymerase, in 25 µl volumes).
3. To check for DNA quality in extract, amplify region of the arthropod mitochondrial nicotinamide adenine dinucleotide dehydrogenase (NADH) subunit 4 (ND4) gene (primers ND4FW [5'-GTD YAT TTA TGA TTR CCT AA-3'] and ND4RV [5'-CTT CGD CTT CCW ADW CGT TC-3']; expect product 400bp)[11,13].
4. To differentiate *Anopheles gambiae sl.* sibling species (*An. gambiae ss* and *An. arabiensis* only), amplify region flanking SNPs in the intergenic spacer (IGS) region, (primers UN [5'-GTGTGCCCCTTCCTCGATGT-3'], GA [5'-CTGGTTTGGTCGGCACGTTT-3'], AR [5'-AAGTGTCCTTCTCCATCCTA-3']; expect product 390bp *An gambiae ss*, 315bp *An arabiensis*)[10,11].
5. For typing *P. falciparum* antifolate drug resistance polymorphisms, perform nested PCR to amplify region flanking amino acid codons 108, 51, 59, 16 and 164 in the parasite dihydrofolate reductase (DHFR) gene:

   Primary round primers: M1 [5'-TTTATGATGGAACAAGTCTGC-3'] and M5 [5'-AGTATATACATCGCTAACAGA-3']

   Secondary round primers: M3 [5'-TTTATGATGGAACAAGTCTGCGACGTT-3'] with F/ [5'-AAATTCTTGATAAACAACGGAAACCTTTTA-3']

   Or

   F [5'-GAAATGTAATTCCCTAGATATGGAATATT-3] with M4 [5'-TTAATTTCCCAAGTAAAACTATTAGAGCTTC-3']).

   Also amplify region containing amino acid codons 436, 437, 540, 581 and 613 in the *P. falciparum* dihydropteroate synthetase (DHPS) gene:

   Primary round primers R2 [5'- AACCTAAACGTGCTGTTCAA-3'] with R/ [5'- AATTGTGTGATTTGTCCACAA3'];

   Secondary round primers

   J: [5'-'TGCTAGTGTTATAGATATAGGTGGAGAAAGC-3'] with K/ [5'-CTATAACGAGGTATTGCATTTATTGCAAGAA-3']

   Or

   K: [5'-TGCTAGTGTTATAGATATAGGATGAGCATC-3'] with K/,

   Or

   L [5'- ATAGGATACTATTTGATATTGATATTGGACCAGGATTCG-3'] with L/ [5'-5TATTACAACATTTTGATCATTCGCGCAACCGG-3'][12].
6. Subject 4 µl amplicon to allele-specific restriction enzyme digestions in 30 µl reactions following the manufacturer's instructions, to detect antifolate drug resistance-associated polymorphisms[12].
7. Subject 5 µl of PCR amplicon (or 15 µl of restriction digest) per lane of ethidium bromide-stained 2% agarose gel to electrophoresis (100 - 120V) and visualize bands under UV transillumination.

Representative Results

Examples of results from PCR assays for mosquito DNA extract quality (**Figure 1**), *Anopheles arabiensis* molecular identification (**Figure 2**) and *P. falciparum* detection (**Figure 3**) show that the simplified Chelex method yields similar results to the standard salting out protocol[10], despite much fewer steps (**Table 1**). With comparable DNA quality in the respective extracts it is not surprising that sample positivity rates with respect to *An. gambiae* sibling species as well as parasite infection rates were not statistically different based on McNemar's chi-square test (**Figure 3**).

Sensitivity (%) was calculated as TP/(TP+FN)*100, where TP denotes true positives and FN denotes false negatives. Specificity (%) was determined as TN/(TN+FP)*100, where TN denotes true negatives and FP denotes false positives. The DNA quality (ND4) PCR showed 93% sensitivity and 82% specificity for the Chelex approach compared to the established salting out protocol as gold standard. Corresponding values of sensitivity and specificity were 100% and 78%, respectively, using sibling species identification PCR and 92% and 80%, respectively for *P. falciparum* detection PCR.

The addition of BSA to reaction mixtures resulted in a general increase of PCR positives (**Figure 3**) due to relief of PCR inhibitors[14], for both the simplified Chelex and regular salting out protocol. However, this increase was not statistically significant except for DNA quality (ND4 PCR) on Chelex extracts ($p = 0.039$). Allele-specific restriction enzyme digestion on *P. falciparum* DHFR (or other target gene) amplicon enables the genotyping of mid-gut and salivary gland malaria infections for drug resistance alleles (**Figure 4; salivary gland data not shown**).

Simple Chelex Procedure		**Standard Salting out Procedure**	
Steps	**Reagents**	**Steps**	**Reagents**
1. Add specimen to 1.5 ml microfuge tube and homogenize in 100 µl of 1X PBS/1% Saponin solution (2 min). 2. Leave at room temperature for 20 min. 3. Spin at 20,000 x g for 2 min and discard supernatant. 4. Add 100 µl of 1X PBS and mix gently by momentarily vortexing (3 sec). 5. Spin at 20,000 x g for 2 min and discard supernatant. 6. Add 25 µl of 20% w/v Chelex in deionized water and 75 µl sterile deionized water. 7. Pierce hole in lid of sample tube using hot, sterile 23G hypodermic needle and boil tube contents for 10 min. 8. Spin microfuge tube at 20,000 x g for 1 min. 9. Transfer supernatant into sterile prelabeled vial and store DNA solution at -20 °C until use (2.5 µl in 25 µl PCR reaction).	PBS Saponin Chelex-100 beads	1. Add specimen to 1.5 ml microfuge tube and homogenize in 100 µl Bender buffer* with 1% DEPC (2 min). 2. Incubate at 65 °C for 1 hr. 3. Add 15 µl cold 8 M potassium acetate. Mix gently and incubate on ice for 45 min. 4. Spin sample in microfuge tubes at 20,000 x g for 10 min and transfer supernatant to a new 1.5 ml tube. 5. Add 250 µl of 100% ethanol and mix well by inverting the tube. 6. Incubate sample at RT for 5 min. 7. Spin samples at 20,000 x g for 15 min. 8. Aspirate and discard supernatant, leaving the pellet to dry completely in the tube (30 min). 9. Resuspend pellet in 20 µl of 0.1X SSC + RNAse (10 µg/ml) overnight at 4 °C. 10. Add 80 µl DEPC water and store at -20 °C until use.	Diethylpyrocarbonate (DEPC) *Bender Buffer: 0.1 M NaCl 0.2 M Sucrose 0.1 M Tris-HCL 0.05 M EDTA, pH 9.1 0.5% SDS in DEPC water 8 M Potassium acetate Ethanol 0.1X Saline sodium citrate (SSC) buffer RNAse (10 µg/ml)
TOTAL TIME: 37 min		TOTAL TIME: 2 hr 47 min, plus overnight	

Table 1. Step by step comparison of the reagents and time requirements for simple Chelex protocol and standard salting out method for DNA extraction from mosquito specimens.

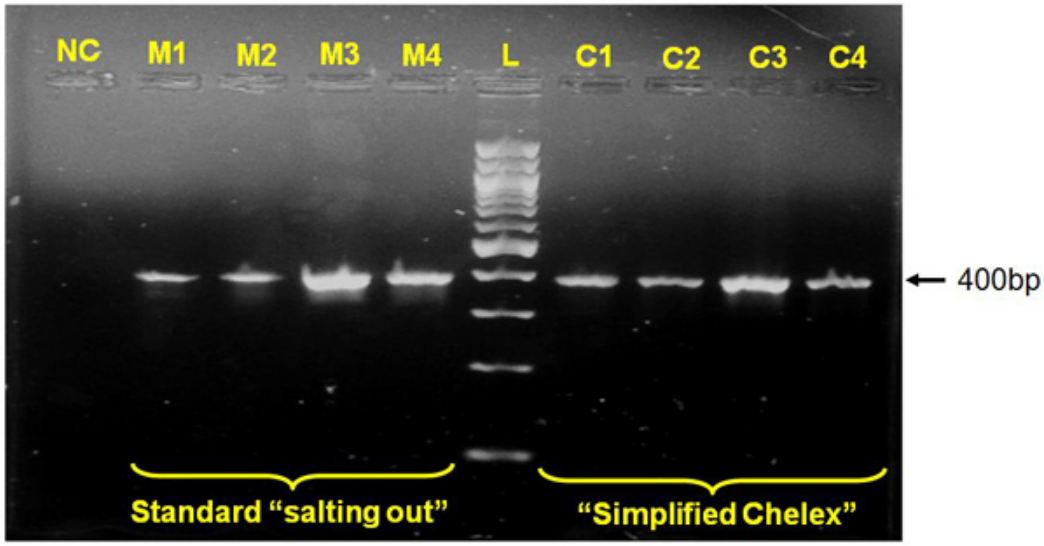

Figure 1. ND4 mitochondrial PCR comparison of DNA quality from simplified Chelex "C" and standard salting out "M" extracts on *Anopheles arabiensis* field samples. NC, negative control; M1 and C1, M2 and C2, M3 and C3, and M4 and C4 are paired salting out and Chelex extracts from same *An. arabiensis* mosquito specimens. L, 100 bp DNA ladder.

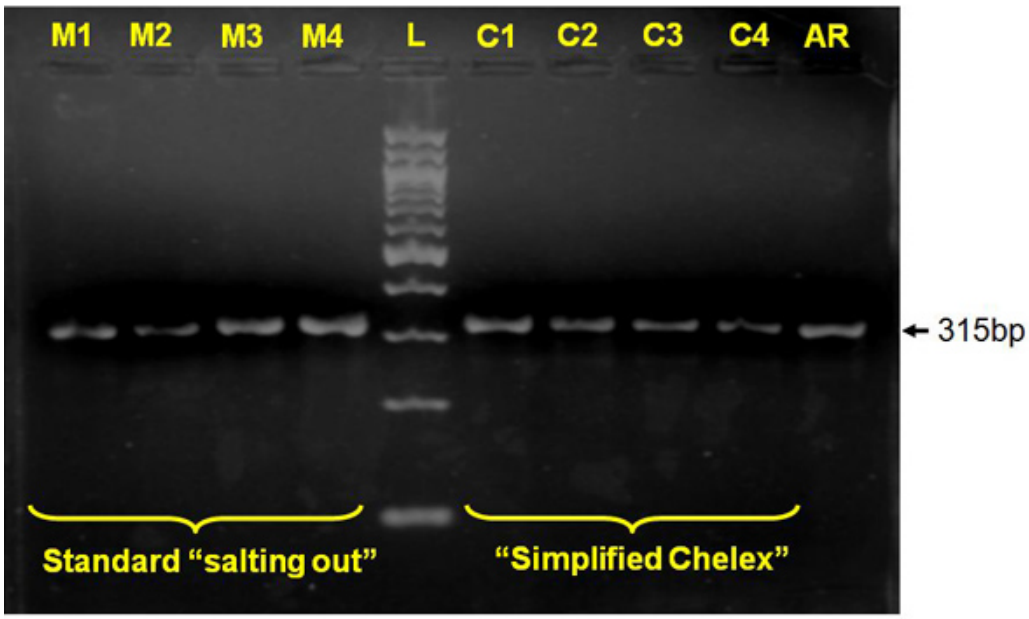

Figure 2. Molecular identification PCR for *Anopheles arabiensis.* C1 and M1, C2 and M2, C3 and M3, C4 and M4 denote respective amplicon from salting out "M" and simplified Chelex "C" DNA extracts for same mosquito specimens; AR, *Anopheles arabiensis* positive control; L, 100 bp DNA ladder.

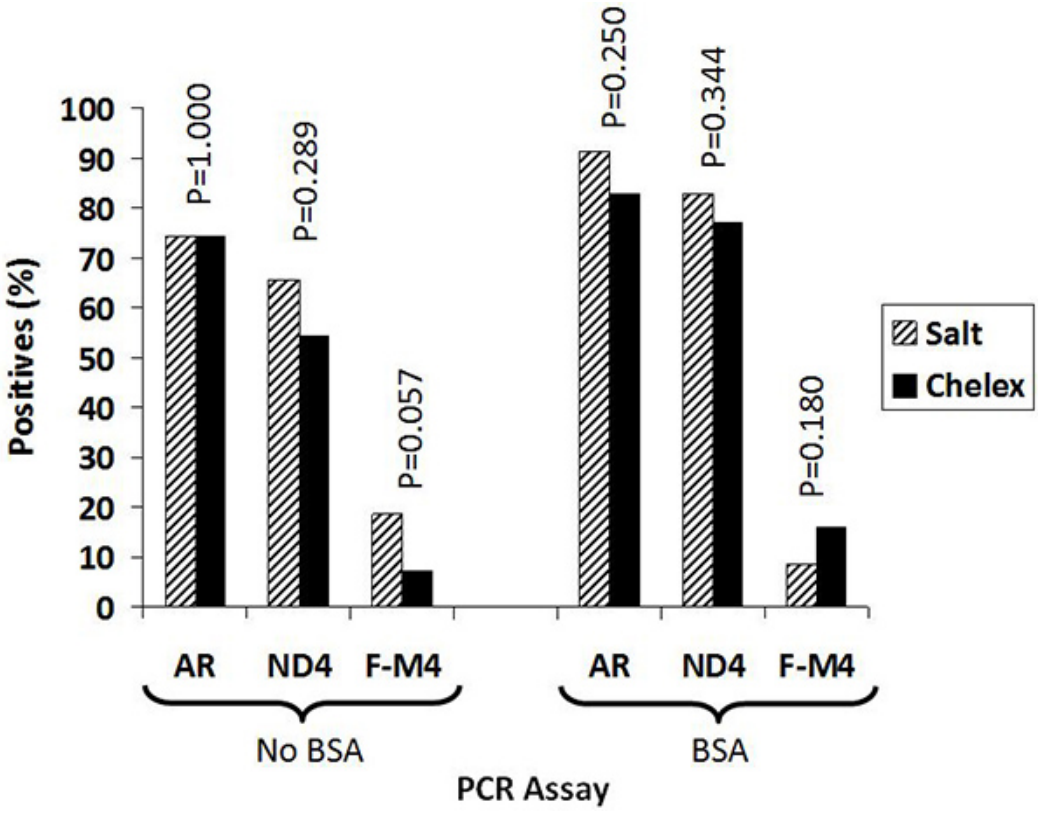

Figure 3. Detection of positives on Chelex and salting out DNA extracts for mosquito field samples, with PCR assays for molecular identification of *Anopheles arabiensis* (AR)[10], arthropod NADH dehydrogenase gene 4 (ND4) DNA quality test[11], and antifolate drug resistance *P. falciparum* DHFR genotyping[12] (F-M4). Results shown for assays run with or without BSA in the reaction mix. McNemar's chi-square test was used to determine if the differences between percent of positive PCRs from DNA extracted from the simplified Chelex and the standard salting out protocols were statistically significant.

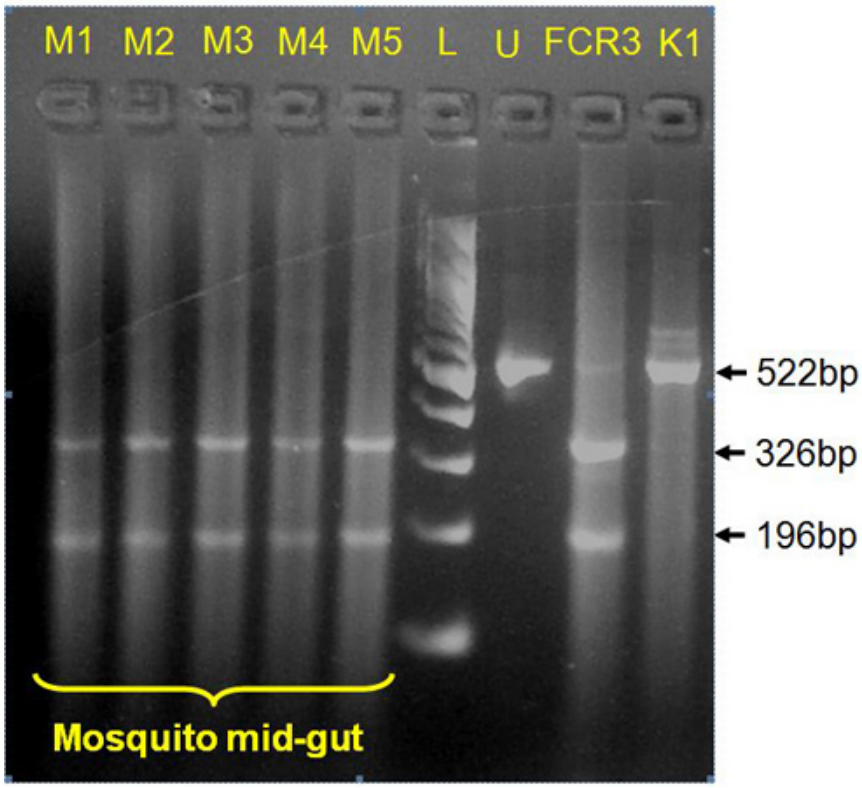

Figure 4. Application of simplified Chelex protocol in genotyping *P. falciparum* infections in mosquitoes (mid-gut data shown) for drug resistance alleles. BstN I digestion on amplicon flanking codon 108 of the *P. falciparum* DHFR gene[12] shows cycloguanil-resistant S108T mutants in mosquito samples (M1-M5; mid-gut data shown). U, undigested 522 bp amplicon; FCR3, laboratory standard *P. falciparum* positive control clone carrying S108T; K1, *P. falciparum* laboratory standard clone negative control carrying cycloguanil-sensitive S108N; L, 100 bp DNA ladder.

Discussion

The simplified Chelex method presented here enables extraction of quality *Anopheles spp* and *P. falciparum* DNA from mosquito specimens amenable to diverse PCR applications. This technique can be employed for molecular identification of malaria vector mosquitoes and surveillance of drug-resistant *P. falciparum* alleles in mosquitoes for national malaria control programs. The advantages of the simplified Chelex technique include simplicity, fewer reagents and hence cost, safety and shorter processing time (37 min) than standard protocols such as the salting out method[10] (2 hr 47 min and an overnight step, **Table 1**). The aforementioned advantages and minimal reagent requirements (3 reagents) compared to the current standard protocol (10 reagents, **Table 1**)[11,15], make the simplified Chelex protocol particularly friendly to endemic country laboratories where a constant reagent supply chain is often difficult to maintain. A limitation of the method is that like the standard protocol, it was also subject to PCR inhibitors known to occur in the mosquito integument[16]. However, this is readily relieved by inclusion of BSA in the assays. BSA has also been successfully employed as an amplification enhancer against inhibitors in other PCR applications[17,18].

Disclosures

No conflicts of interest declared.

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